Data analysis supplement to the research article Abouelezz, Stefen, Segerstråle, Micinski, Minkeviciene, Lahti, Hardeman, Gunning, Hoogenraad, Taira, Fath, & Hotulainen (2020), "Tropomyosin Tpm3.1 Is Required to Maintain the Structure and Function of the Axon Initial Segment"

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Abstract: Relying on previous publications and tools (Bob, 2010; Costes et al., 2004; Dunn et al., 2011; Fiji/ImageJ program web site, 2020; Herbert, undated; Fiji/ImageJ program documentation, 2020; Online Manual for the WCIF-ImageJ collection, undated; Stauffer et al. 2018), this current document describes a method developed by Lauri Lahti and how he used this method to perform the co-localization analysis and create the results that are reported in the research article Abouelezz, Stefen, Segerstråle, Micinski, Minkeviciene, Lahti, Hardeman, Gunning, Hoogenraad, Taira, Fath, & Hotulainen (2020), "Tropomyosin Tpm3.1 Is Required to Maintain the Structure and Function of the Axon Initial Segment".

Introduction

Relying on previous publications and tools (Bob, 2010; Costes et al., 2004; Dunn et al., 2011; Fiji/ImageJ program web site, 2020; Herbert, undated; Fiji/ImageJ program documentation, 2020; Online Manual for the WCIF-ImageJ collection, undated; Stauffer et al. 2018), this current document describes a method developed by Lauri Lahti and how he used this method to perform the co-localization analysis and create the results that are reported in the following peer-reviewed scientific journal article:
Abouelezz, Amr; Stefen, Holly; Segerstråle, Mikael; Micinski, David; Minkeviciene, Rimante; Lahti, Lauri; Hardeman, Edna C.; Gunning, Peter W.; Hoogenraad, Casper C.; Taira, Tomi; Fath, Thomas; & Hotulainen, Pirta (2020). Tropomyosin Tpm3.1 is required to maintain the structure and function of the axon initial segment. iScience. 2020 Apr 12;23(5):101053. DOI: 10.1016/j.isci.2020.101053. https://www.sciencedirect.com/science/article/pii/S2589004220302388 and https://doi.org/10.1016/j.isci.2020.101053

Lauri Lahti's author contributions for the above-mentioned journal article (Abouelezz et al., 2020) include that he performed the co-localization analysis and also participated in writing, reviewing, commenting and editing the manuscript. Among other things, the methods and results of the journal article (Abouelezz et al., 2020) rely on measuring properties of the membrane periodic skeleton of the axon initial segment using cultured rat hippocampal neurons and 3D-structured illumination microscopy (3D-SIM).

Method for performing the co-localization analysis concerning properties of the membrane periodic skeleton of the axon initial segment using cultured rat hippocampal neurons and 3D-structured illumination microscopy (3D-SIM)

This method is developed by Lauri Lahti. This method is descripted in this current document written by Lauri Lahti, completed and self-archived on 10 September 2021. The first version of this document was completed on 10 February 2020.
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This description of the method relies on previous publications and tools (Bob, 2010; Costes et al., 2004; Dunn et al., 2011; Fiji/ImageJ program web site, 2020; Herbert, undated; Fiji/ImageJ program documentation, 2020; Online Manual for the WCIF-ImageJ collection, undated; Stauffer et al. 2018).

1. Launching the Fiji/ImageJ program.

Ensure that you have Fiji/ImageJ program (https://imagej.nih.gov/ij/) installed on your computer environment. Then launch the Fiji/ImageJ program.

2. Open a hyperstack image file (a hyperstack image file that contains three color channels and each of these three channels has a series of images (for example 33 images) taken along the z axis).

In the main command panel window of the Fiji/ImageJ program, select the command path:
File -> Open… ->
Select the file name. Let's assume that we select now file name "phalloidin-ankG-gamma9d-10_copy.tif".
Press "Open" button.

A new window (titled "phalloidin-ankG-gamma9d-10_copy.tif") should open containing the hyperstack image.

3. Split the hyperstack image so that each of three color channels is shown as a separate image stack.

Select the command path: Image -> Color -> Split Channels

Three new windows (titled "C1-phalloidin-ankG-gamma9d-10_copy.tif", "C2-phalloidin-ankG-gamma9d-10_copy.tif" and "C3-phalloidin-ankG-gamma9d-10_copy.tif") should open, each of them showing an image stack (33 frames) for a color channel.

4. Identify "the sharpest/brightest" image frames that should be included into the new "z projection by max intensity" image versions.

a)

Let's assume that the channel 2 holds now the most important signal for identifying the region of interest for the colocalization analysis concerning the comparison of the signals between the three channels of the hyperstack image.
Thus we now start with the channel 2 image frames (shown in the window titled "C2-phalloidin-ankG-gamma9d-10_copy.tif") to identify "the sharpest/brightest" image frames in channel 2.

b)

By sliding the scroll bar in the bottom of the window “C2-phalloidin-ankG-gamma9d-10_copy.tif” we can browse through the images taken along z axis for channel 2. We can see how in a certain relatively narrow range of consecutive frames the image holds “the sharpest/brightest” signal.
Let’s assume that “the sharpest/brightest” signal of channel 2 emerges in the range of frames 17-19 (i.e. this range includes the frame 17, the frame 18 and the frame 19).

Before performing the following command path please make sure that the window “C2-phalloidin-ankG-gamma9d-10_copy.tif” is currently the most recently active window (if you are unsure, click on the title bar of the window “C2-phalloidin-ankG-gamma9d-10_copy.tif” to make it active and do not click on any other window before proceeding to the following command path).
Select the command path: Image -> Stacks -> Z Project…

A new window (titled “Zprojection”) should open.

Useful settings:
Start slice: 17
Stop slice: 19
Projection type: Max intensity
Press “OK” button.

A new window (titled “MAX_C2-phalloidin-ankG-gamma9d-10_copy.tif”) should open.

c)

As mentioned already in “the step 4a” above let’s indeed assume that the channel 2 holds now the most important signal for identifying the region of interest for the colocalization analysis concerning the comparison of the signals between the three channels of the hyperstack image.

Thus based on “the step 4b” we should now already know where is approximately the region of interest for the hyperstack image also concerning the channel 1 and channel 3.

By sliding the scroll bar in the bottom of the window “C1-phalloidin-ankG-gamma9d-10_copy.tif” we can browse through the images taken along z axis for channel 1. We try to identify those frames that hold “the sharpest/brightest” signal especially in the region of interest that we already identified in “the step 4b”.

Let’s assume that “the sharpest/brightest” signal of channel 1 (in the desired region of interest based on channel 2) emerges in the range of frames 16-18. Note that this range of frames for channel 1 (16-18) is different than the range of frames for the channel 2 (17-19).

Before performing the following command path please make sure that the window “C1-phalloidin-ankG-gamma9d-10_copy.tif” is currently the most recently active window (if you are unsure, click on the title bar of the window “C1-phalloidin-ankG-gamma9d-10_copy.tif” to make it active and do not click on any other window before proceeding to the following command path).
Select the command path: Image -> Stacks -> Z Project…

A new window (titled “Zprojection”) should open.

Useful settings:
Start slice: 16
Stop slice: 18
Projection type: Max intensity
Press “OK” button.

A new window (titled “MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif”) should open.

d)

Similarly as was done in “the step 4c” for the channel 1, now let’s identify the range of “the sharpest/brightest” image frames for channel 3.

By sliding the scroll bar in the bottom of the window “C3-phalloidin-ankG-gamma9d-10_copy.tif” we can browse through the images taken along z axis for channel 3. We try to identify those frames that hold “the sharpest/brightest” signal especially in the region of interest that we already identified in “the step 4b”.

Let’s assume that “the sharpest/brightest” signal of channel 3 (in the desired region of interest based on channel 2) emerges in the range of frames 15-17. Note that this range of frames for channel 3 (15-17) is different than the range of frames for the channel 2 (17-19) and also different than the range of frames for the channel 1 (16-18).

Before performing the following command path please make sure that the window “C3-phalloidin-ankG-gamma9d-10_copy.tif” is currently the most recently active window (if you are unsure, click on the title bar of the window “C3-phalloidin-ankG-gamma9d-10_copy.tif” to make it active and do not click on any other window before proceeding to the following command path).
Select the command path: Image -> Stacks -> Z Project…

A new window (titled “Zprojection”) should open.

Useful settings:
Start slice: 15
Stop slice: 17
Projection type: Max intensity
Press “OK” button.

A new window (titled “MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif”) should open.

5. Save the three separate “z projection by max intensity” image versions.

a)

Make sure that the window “MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif” is currently the most recently active window (if you are unsure, click on the title bar of the window “MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif” to make it active and do not click on any other window before proceeding to the following command path)
.
Select the command path: File -> Save as -> Tiff…

Select a desired file folder (preferably a specific new folder, such as “channel_1_z_proj_by_max_int”).
Use the default file name: MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif
Press “Save” button.

b)

Make sure that the window “MAX_C2-phalloidin-ankG-gamma9d-10_copy.tif” is currently the most recently active window (if you are unsure, click on the title bar of the window “MAX_C2-phalloidin-ankG-gamma9d-10_copy.tif” to make it active and do not click on any other window before proceeding to the following command path)
.
Select the command path: File -> Save as -> Tiff…

Select a desired file folder (preferably a specific new folder, such as “channel_2_z_proj_by_max_int”).
Use the default file name: MAX_C2-phalloidin-ankG-gamma9d-10_copy.tif
Press “Save” button.

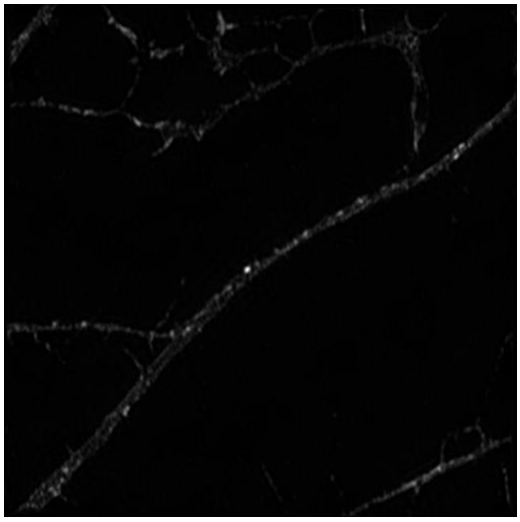
c)

Make sure that the window “MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif” is currently the most recently active window (if you are unsure, click on the title bar of the window “MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif” to make it active and do not click on any other window before proceeding to the following command path).

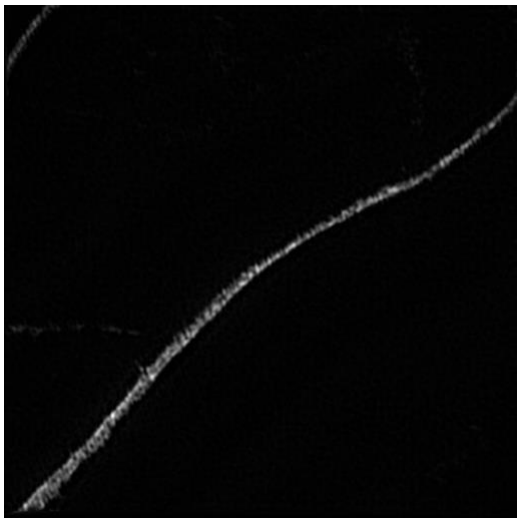
.

Select the command path: File -> Save as -> Tiff…

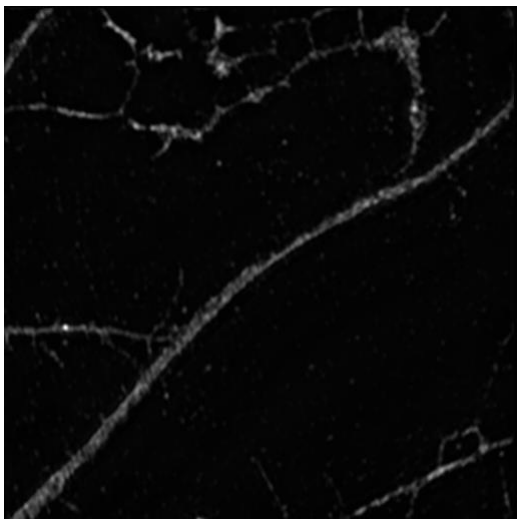
Select a desired file folder (preferably a specific new folder, such as “channel_3_z_proj_by_max_int”).
Use the default file name: MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif
Press “Save” button.



MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif



MAX_C2-phalloidin-ankG-gamma9d-10_copy.tif


MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif

6. Create a composite image based on the three separate "z projection by max intensity" image versions that were created in "the step 5".

We assume that based on "the step 5" the windows "MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif", "MAX_C2-phalloidin-ankG-gamma9d-10_copy.tif" and "MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif" are still open.
(If needed, these three images can be opened again with the command path: File – Open… -> etc.)

If any other windows than those three above mentioned windows are currently open, please close them now first to prevent a possible malfunctioning.

Select the command path: Image -> Stacks -> Images to stack

Useful settings:
Name: Stack (the default value)
Title Contains: (the default value is empty)
Use Titles as Labels: selected option (checkbox selected)
Keep Source Images: selected option (checkbox selected)
Press "OK" button.

A new window (titled "Stack") should open.
(Note: For some reason, now sliding the scrollbar of the window "Stack" does not seem to function as expected (i.e. the three separate channel images do not seem to appear as expected). Despite of this strange behavior anyway in the following actions it seems that the composite image correctly includes the separate channel images.)

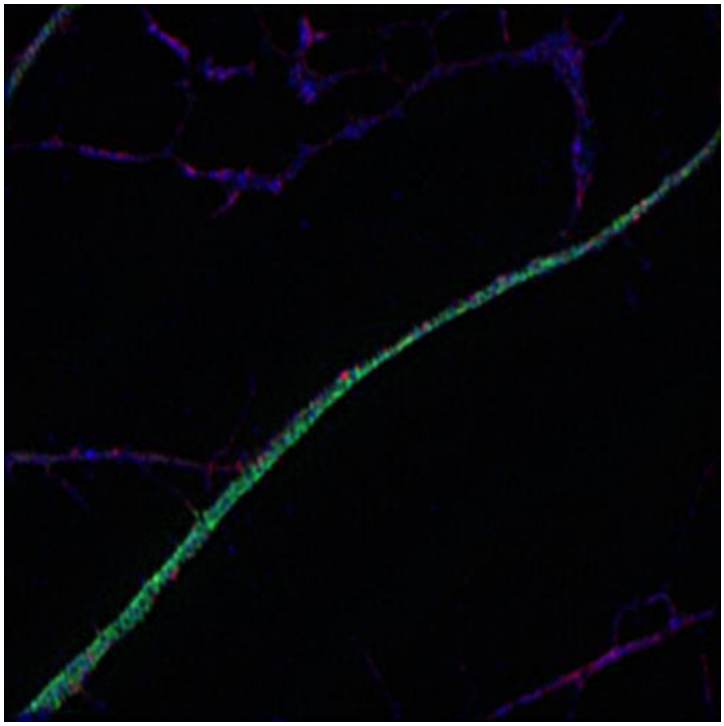
Make sure that the window "Stack" is currently the most recently active window (if you are unsure, click on the title bar of the window "Stack" to make it active and do not click on any other window before proceeding to the following command path).

Select the command path: Image -> Color -> Make composite

The outlook of the window "Stack" should now change so that it shows a composite image that indicates with three different colors the signals of the three channels (channel 1 indicated with red color, channel 2 indicated with green color and channel 3 indicated with blue color).

Make sure that the window "Stack" is currently the most recently active window (if you are unsure, click on the title bar of the window "Stack" to make it active and do not click on any other window before proceeding to the following command path).

Select the command path: File -> Save as -> Tiff…
Select a desired file folder (preferably a specific new folder, such as “composite_z_proj_by_max_int”).
Use a file name that matches the original source file names:
phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite.tif
Press “Save” button.


phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite.tif

7. With a separate drawing program (for example the Gimp program, see https://www.gimp.org/) create an outline image defining the region of interest for colocalization analysis.

How an outline image defining the region of interest can be created with the Gimp program:

In the Gimp program, select the command path: File -> Open…
Select a desired file folder containing the appropriate composite image that was created in “the step 6” (let’s assume that this folder is “composite_z_proj_by_max_int”).
Select the file name of the appropriate composite image, for example “phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite.tif”.
Press “Open” button.
The composite image should appear into the editing area of the Gimp program (channel 1 indicated with red color, channel 2 indicated with green color and channel 3 indicated with blue color).

As mentioned already in “the step 4a” above let’s indeed assume that the channel 2 holds now the most important signal for identifying the region of interest for the colocalization analysis concerning the comparison of the signals between the three channels of the hyperstack image. Since in the composite image the channel 2 is indicated with the green color, we can now create a white outline image defining the region of interest for colocalization analysis so that we draw a thin white line approximately along the outer borders of the green color patterns (approximately means here that we try to make sure that relevant bright pixel areas also in the other channels 1 and 3 can be captured inside the white outline). To make this white outline image it is possible to do the following actions:

Important:
To prevent malfunctioning and possible error messages in the later launching of “EzColocalization” with Analyze button it is important to ensure that the white drawn outline area never touches the outermost borders of the image, instead there must always be at least one pixel of black color on the outer edge of the image’s borders.

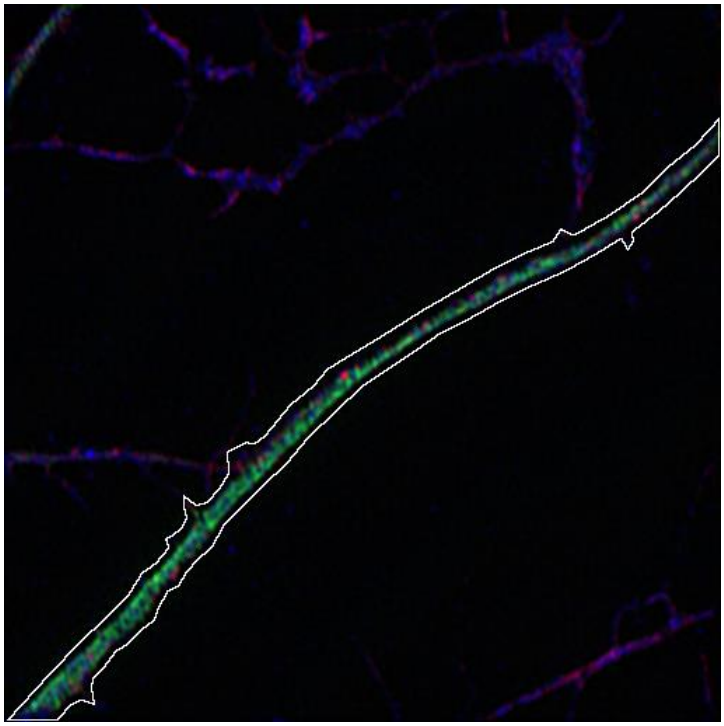
The final “outline image” should have the region of interest painted with white color pixels on a black background.
In the Gimp program, select the command path: Layer -> New Layer… -> (Fill with: Transparency) -> OK
Draw a white line on the new layer with the Pencil Tool: First click with the mouse on a suitable place on the image to create a starting point for a line and then add new line segments so that for the following clicks keep also the shift button pressed down. The white line should form a closed loop and then it should be filled with white color by using he Bucket Fill Tool. Thus the final “outline image” should contain a region of white color pixels (i.e. the region of interest) that is on a black background.

The outline image can be exported in the Gimp program by selecting the command path: File -> Export as…

It is practical to export two different output versions: a) “outline layered” and b) “only outline with inside filled”.

Case 1/2: Before the inside of the outlined area is filled with white color by using the Bucket Fill Tool:

a) Exporting "outline layered" image:
It can be practical to export now to png image format.
Keep visible all layers of the image.
Select a desired file folder (preferably such as "composite_z_proj_by_max_int").
Select the file name, for example "phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_layered.png".
Press "Export" button, etc.


phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_layered.png

Case 2/2. After the inside of the outlined area is filled with white color by using the Bucket Fill Tool:

b) Exporting "only outline with inside filled" image:
It is important to export now to tiff image format.
Keep visible only the layer that holds the white color pixels that define the region of interest.
Select a desired file folder (preferably such as "composite_z_proj_by_max_int").
Select the file name, for example "phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif".
Press "Export" button, etc.

Please note: Due to some possible format encoding challenges or settings it might turn out that the images exported from Gimp program might not always open as expected in the Fiji/ImageJ program. Anyway, the essential aim is to create and export *an "only outline with inside filled" image* that indicates the region of interest with white color pixels on a black background.

phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif

8. Install the Fiji/ImageJ "EzColocalization Plugin".

Install the Fiji/ImageJ "EzColocalization Plugin" that is available from:
Stauffer, W., Sheng, H., and Lim, H.N. (2018). EzColocalization: An ImageJ plugin for visualizing and measuring colocalization in cells and organisms. See https://www.nature.com/articles/s41598-018-33592-8 and https://sites.imagej.net/EzColocalization/plugins/

9. Make colocalization analysis with the Fiji/ImageJ "EzColocalization Plugin"

The colocalization analysis enables comparing the signals between two channels of the hyperstack image in a "region of interest" defined by the outline image.

Let's assume that we are now observing the data originating from the file "phalloidin-ankG-gamma9d-10_copy.tif".
We want to compare signals between channel 1 and channel 3 in the "region of interest" defined by the outline image. Thus we first open three appropriate images.

In the Fiji/ImageJ program do the following actions:

To prevent malfunctioning and errors, please preferably first close all currently open windows except the main command panel window of the Fiji/ImageJ program.

Important:
It is recommendable to make sure that the "EzColocalization" window is not yet opened before opening any image files. If the "EzColocalization" window is already open before the image files are opened, it seems that some settings of the "EzColocalization" window do appear only partially available.

How to open images for "EzColocalization"?

RECOMMENDED: It seem to be preferable to open the images through the traditional drop-down "File menu" that is located in the main command panel window of the Fiji/ImageJ program.

(NOT RECOMMENDED: It seems that one suggested practice for opening the image files for the "EzColocalization Plugin" is to do it through the drop-down "File menu" that is located in the "EzColocalization" window (a separate window that shows the adjustable settings for the "EzColocalization Plugin". Unfortunaltely using this strategy seems to cause sometimes that some settings of the "EzColocalization" window do appear only partially available.)

a) Open the "z projection by max intensity" image version of channel 1

Through the traditional drop-down "File menu" that is located in the main command panel window of the Fiji/ImageJ program, select the command path:
File -> Open...
Select a desired file folder (for example "channel_1_z_proj_by_max_int", like in "the step 5").
Select a file name, for example MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif
Press "Open" button.

b) Open the "z projection by max intensity" image version of channel 3

Through the traditional drop-down "File menu" that is located in the main command panel window of the Fiji/ImageJ program, select the command path:
File -> Open...
Select a desired file folder (for example "channel_3_z_proj_by_max_int", like in "the step 5").
Select a file name, for example MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif
Press "Open" button.

c) Open the "outline image" that defines the region of interest (illustrated with a region of white color pixels on a black background)

Through the traditional drop-down "File menu" that is located in the main command panel window of the Fiji/ImageJ program, select the command path:
File -> Open...
Select a desired file folder (for example "composite_z_proj_by_max_int", like in "the step 7").
Select the file name, for example "phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif".
Press "Open" button.

Important:
As already mentioned in "the step 7", please note this specific requirement for the "outline image": To prevent malfunctioning and possible error messages in the later launching of "EzColocalization" with Analyze button it is important to ensure that the white drawn outline area never touches the outermost borders of the image, instead there must always be at least one pixel of black color on the outer edge of the image's borders.

If along the steps a), b) and c) there appears a warning message "The calibration of this image conflicts with the current global calibration", then select the following settings in the warning window:

Selected checkbox: Disable Global Calibration
Not selected checkbox: Disable these Messages
Finally press "OK" button.

d) The "outline image" must be converted to be a mask

Do the conversion in the following way:

Make sure that the window "phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif" is currently the most recently active window
(if you are unsure, click on the title bar of the window "Stack" to make it active and do not click on any other window before proceeding to the following command path).

In the main command panel window of the Fiji/ImageJ program, select the command path:
Process -> Binary -> Convert to mask

e) Scaling factors must be made consistent/global

Note: This action is important, if this is not performed, the later launching of "EzColocalization" with Analyze button will bring an error message: Scaling factors must be consistent/global. Reset the Scale in "Analysis -> Set scale…".

In the main command panel window of the Fiji/ImageJ program, select the command path:
Analysis -> Set scale… ->
Press button "Click to remove scale"
Select option "Global"
Press button "OK".

Thus the following values were accepted by pressing button "OK":
Distance in pixels: 0.00
Know distance: 0.00
Pixel aspect ratio: 1.0
Unit of length: pixel
Selected checkbox: "Global"
Scale: <no scale>

f) Launch "EzColocalization Plugin"

In the main command panel window of the Fiji/ImageJ program, select the command path:
Plugins -> EzColocalization

A new window titled "EzColocalization" should appear.

g) Adjust the settings in the "Inputs tab" of the "EzColocalization" window

Note: In the "Inputs tab" of the "EzColocalization" window there are settings that define "Reporter 1 (Ch. 1)", "Reporter 2 (Ch. 2)" and "Cell identification input". Here the notations Ch. 1 and Ch. 2 do not necessarily refer to channels 1 and 2 specifically but instead two different channels that the user can select among various channel alternatives according to his/her will. Thus also in the following guidance example you can see that the settings for "Reporter 2 (Ch. 2)" are used to refer to channel 3 of the hyperstack image.

Some settings in the "Inputs tab" of the "EzColocalization" window should become automatically updated.
If the settings that define "Reporter 1 (Ch. 1)", "Reporter 2 (Ch. 2)" and "Cell identification input" become automatically updated in a wrong way, you can manually ensure that the settings are like this in the "Inputs tab":

Images for analysis:
Reporter 1 (Ch. 1): MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif

Reporter 2 (Ch. 2): MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif
Cell identification input: phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif

Alignment and threshold options:
Not selected checkbox: Align Reporter 1 (Ch. 1): Default
Not selected checkbox: Align Reporter 2 (Ch. 2): Default

h) Keep the default settings in the "Cell Filters tab" of the "EzColocalization" window

Ensure that the settings are like this in the "Cell Filters tab":

Area: 0-Infinity

Not selected checkbox: Watershed segmentation

Cell filter 1 Area 0-Infinity
Cell filter 2 AR 0-Infinity
Cell filter 3 Mean (Ch.1) 0-Infinity
Cell filter 4 Mean (Ch.2) 0-Infinity
Cell filter 5 Min (Ch.1) 0-Infinity
Cell filter 6 Min (Ch.2) 0-Infinity
Cell filter 7 Max (Ch.1) 0-Infinity
Cell filter 8 Max (Ch.2) 0-Infinity

i) Adjust the settings in the "Visualization tab" of the "EzColocalization" window

Adjust the settings in the "Visualization tab" of the "EzColocalization" window to be like this:

Heat maps
Scaling options:
Selected radio button option: cell
Color maps:
Selected checkbox: Channel 1 hot
Selected checkbox: Channel 2 cool

Scatterplots
Selected checkbox: Cell pixel intensity scatterplots

Metric matrices
Selected checkbox: Matrices of different combinations of fractions
FT (Ch.1): 10 %
FT (Ch.2): 10 %
Metric: PCC
Average: Median

j) Adjust the settings in the "Analysis tab" of the "EzColocalization" window

Adjust the settings in the "Analysis tab" of the "EzColocalization" window to be like this:

In the "Analysis Metrics sub-tab":

Colocalization metrics:
Selected checkbox: TOS, also selected radio button "Costes"'
Selected checkbox: PCC, also selected radio button "All"

Selected checkbox: SRCC, also selected radio button "All"
Selected checkbox: ICQ, also selected radio button "All"
Selected checkbox: MCC, also selected radio button "Costes"'

Other metrics:
Selected checkbox: Average signal
Not selected checkbox: Custom Metric
Selected checkbox: Summary
Not selected checkbox: Histogram(s)
Not selected checkbox: Mask(s)
Not selected checkbox: ROI(s)

In the "Metrics info sub-tab":
Keep the default settings.

In the "Custom sub-tab":
Keep the default settings.

10. Launch calculations of the "EzColocalization" window.

In the bottom of the “EzColocalization” window press the button “Analyze”. This should launch calculations and. Based on the above mentioned settings, two specific results windows should appear: a window titled “Metric(s) of…” and a window titled “Log”. Also some supplementing visualization results should appear in additional windows.

a)

The window “Metric(s) of…” should provide numerical results that are like the following ones:

Label	TOS(linear)	TOS(log2)	PCC	SRCC	ICQ	M1	M2
image 1: cell 1	NaN	NaN	0.605	0.754	0.329	0.979	1

Avg.Int.C1	Avg.Int.C2	Area	X	Y	XM	YM	Perim.
4299.831	8491.970	16032	239.993	298.688	239.993	298.688	1484.844

BX	BY	Width	Height	Major	Minor	Angle	Circ.
2	81	509	430	474.109	43.055	39.626	0.091

Feret	FeretX	FeretY	FeretAngle	MinFeret	AR	Round	Solidity
666.319	2	511	40.191	77.103	11.012	0.091	0.431

b)

The window “Log” should provide textual results that are like the following ones:

Results Summary:

Reporter 1 (Ch.1) image(s): MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif (Unaligned)
Reporter 2 (Ch.2) image(s): MAX_C3-phalloidin-ankG-gamma9d-10_copy.tif (Unaligned)
Cell identification input image(s): phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif

Number of cells analyzed = 1

Threshold Overlap Score linearly rescaled [TOS(linear)]

mean = NaN
standard deviation = NaN
median = NaN

Interpretation:
TOS(linear) is a measure of the overlap of pixels above the threshold for two or three reporter channels, normalized for the expected overlap to occur by chance, which is rescaled so the value is a fraction of the difference between the null hypothesis and the maximum possible colocalization or anticolocalization for the selected thresholds.
For example, 0.5 is halfway between the null distribution and complete overlap for the selected percentages.

-1 = complete anticolocalization
0 = noncolocalization
1 = complete colocalization

Threshold Overlap Score logarithmically rescaled [TOS(log2)]

mean = NaN
standard deviation = NaN
median = NaN

Interpretation:
TOS(log) is the same as TOS(linear) except the rescaling is logarithmic instead of linear.
For example: 0.5 is halfway between the null distribution and complete overlap of selected percentages on the log scale.

-1 = complete anticolocalization
0 = noncolocalization
1 = complete colocalization

Pearson's Correlation Coefficient (PCC)

mean = 0.6054364144631682
standard deviation = 0.0
median = 0.6054364144631682

Interpretation:
PCC measures the correlation between the pixel values for two reporter channels.

-1 = complete anticolocalization
0 = noncolocalization
1 = complete colocalization

Spearman's Rank Correlation Coefficient (SRCC)

mean = 0.7542064512612362
standard deviation = 0.0
median = 0.7542064512612362

Interpretation:
SRCC measures the ranked correlation of pixel values for two reporter channels.

-1 = complete anticolocalization
0 = noncolocalization
1 = complete colocalization

Intensity Correlation Quotient (ICQ)

mean = 0.32865518962075846
standard deviation = 0.0
median = 0.32865518962075846

Interpretation:
ICQ measures the proportion of pixels that are below or above the mean for all of two or three reporter channels.

-0.5 = complete anticolocalization
0 = noncolocalization
0.5 = complete colocalization

Manders' Colocalization Coefficient (MCC; components M1, M2, and M3) of Channel 1

mean = 0.9790980173524887
standard deviation = 0.0
median = 0.979098017352488 7

Interpretation:
MCC measures the intensity weighted proportion of signal which overlaps above the thresholds for two or three channels.

0 = complete anticolocalization
1 = complete colocalization

Manders' Colocalization Coefficient (MCC; components M1, M2, and M3) of Channel 2

mean = 1.0
standard deviation = 0.0
median = 1.0

Interpretation:
MCC measures the intensity weighted proportion of signal which overlaps above the thresholds for two or three channels.

0 = complete anticolocalization
1 = complete colocalization

Average Signal Intensity (Avg.Int.) of Channel 1

mean = 4299.8313373253495
standard deviation = 0.0
median = 4299.8313373253495

Interpretation:
Selection of the average signal intensity option generates a table that has the average signal intensity of each reporter channel and the physical measurements of each cell in the sample.

Average Signal Intensity (Avg.Int.) of Channel 2

mean = 8491.969810379242
standard deviation = 0.0

median = 8491.969810379242

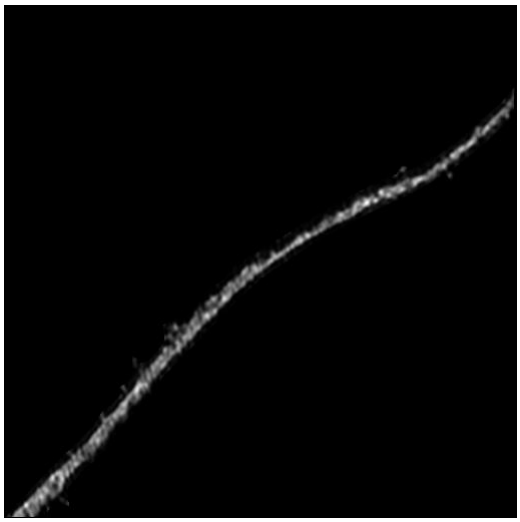
Interpretation:
Selection of the average signal intensity option generates a table that has the average signal intensity of each reporter channel and the physical measurements of each cell in the sample.

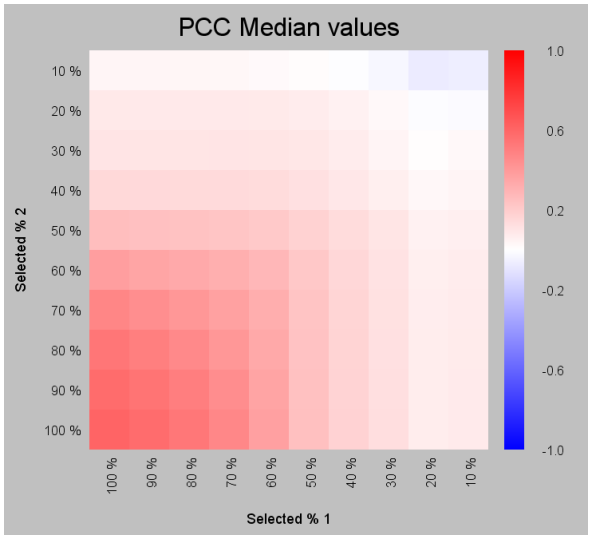
---

c)

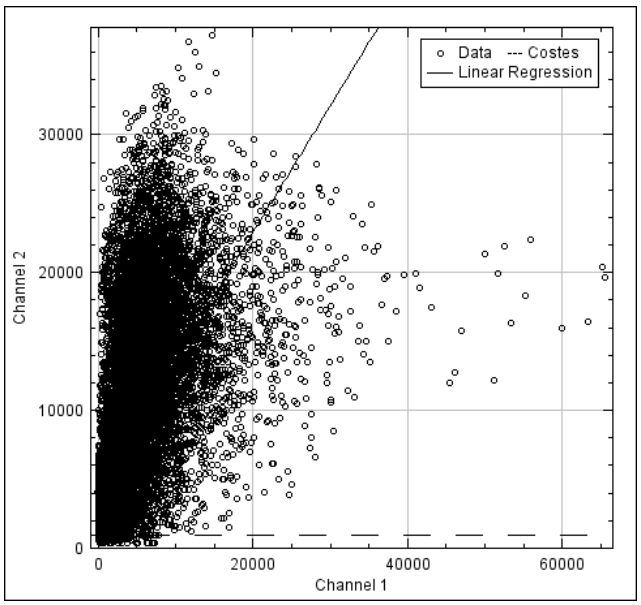
Some supplementing visualization results should appear like the following ones:


Heatmap(s) of MAX_C1-phalloidin-ankG-gamma9d-10_copy.tif-2.tif


Heatmap(s) of MAX_ C3-phalloidin-ankG-gamma9d-10_copy.tif-2.tif



PCC of phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled.tif



Scatterplots of random cells in phalloidin-ankG-gamma9d-10_copy_stack_ch123_composite_outline_filled_modified.tif

11. Exporting numeric values and visualization results for further use.

The numeric values generated into the window titled "Metric(s) of..." and into the window titled "Log" can be copied to a spreadsheet program or statistics program to perform further analysis.

The generated visualization results can be saved with a specific script (sometimes the saved images can become a bit distorted or some of them may not become saved at all, then try to save images manually, for example by taking screen shots):

a)

Save the following script code to a new text file (let's assume that the file name is "saving_script_by_lauri_lahti_20200210.txt":

```
dir = getDirectory("Choose a Directory");
ids=newArray(nImages);
for (i=0;i<nImages;i++) {
	selectImage(i+1);
	title = getTitle;
	print(title);
	ids[i]=getImageID;

	saveAs("tiff", dir+title);
}
```

b)

To launch the above mentioned saving script:

In the main command panel window of the Fiji/ImageJ program, select the command path:
Plugins -> Macros -> Run...
Select a desired file folder (the folder that holds the script "saving_script_by_lauri_lahti_20200210.txt").
Select the file name. In the following we assume that we use the file name "saving_script_by_lauri_lahti_20200210.txt".
Press "Open" button.

A new window (titled "Choose a Directory") should open.
Now select the desired output folder for saving the visualization results.
Press button "Select".

Visualization results should become now saved into the selected output folder.
(Sometimes the saved images can become a bit distorted or some of them may not become saved at all, then try to save images manually, for example by taking screen shots).

12. Starting a new round for analyzing colocalization

Now it is possible to start a new round for analyzing colocalization in the Fiji/ImageJ program.
You can start repeating “the steps 6-11” described above but still before that note the following:
To prevent malfunctioning and errors, please preferably first close all currently open windows except the main command panel window of the Fiji/ImageJ program.
Thus please now close all still open image windows, "EzColocalization" window, the window "Metric(s) of...", the window "Log" and all visualization results.
Then you can proceed back to “the step 6” and start a new round for analyzing colocalization.

13. References

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